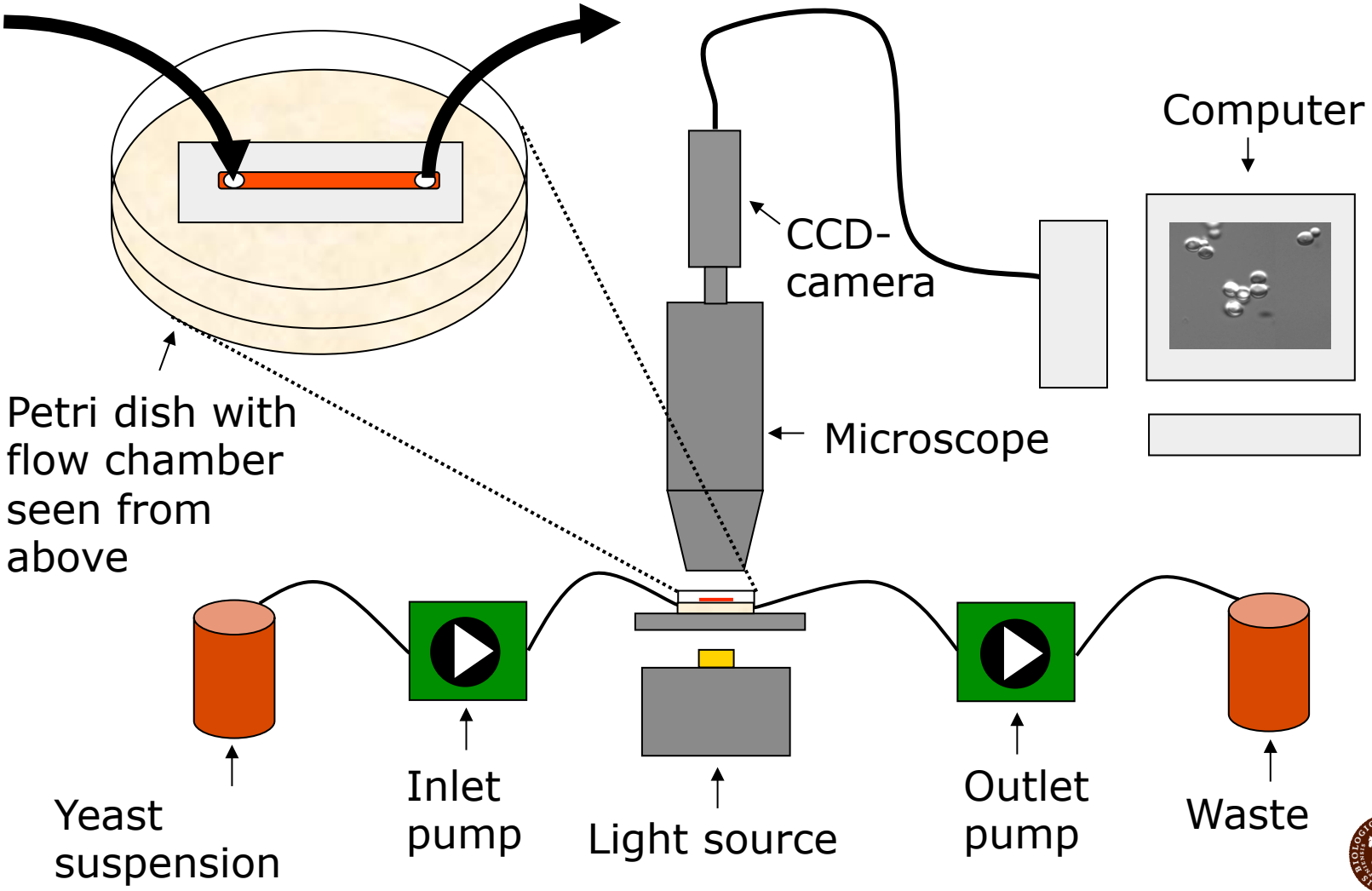
Adhesion assays

- **Henrik Siegumfeldt**

(but most of the work has been performed by colleagues)

Experimental setup of adhesion assay



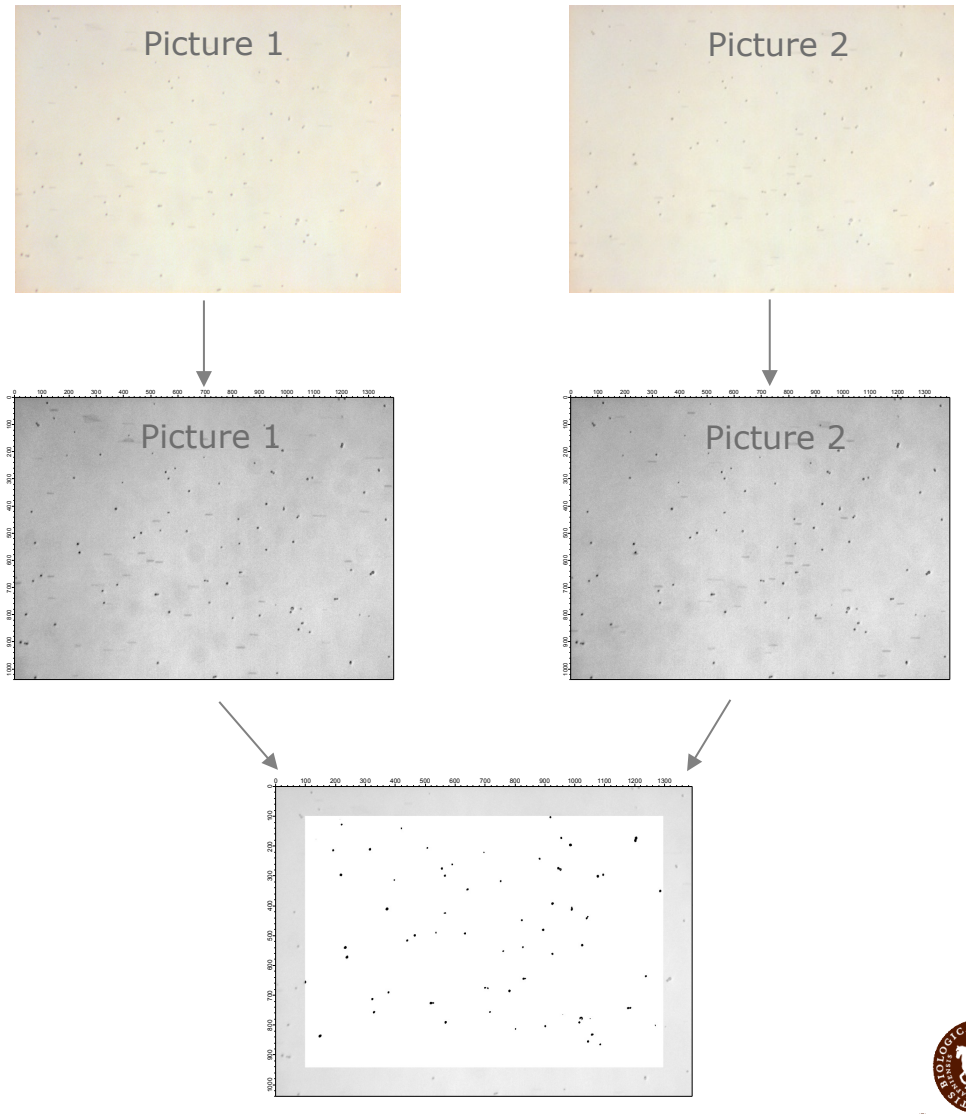
Adhesion of *Debaryomyces hansenii* (CBS 767) on an agarose surface (20 min)

Data analysis of digital images

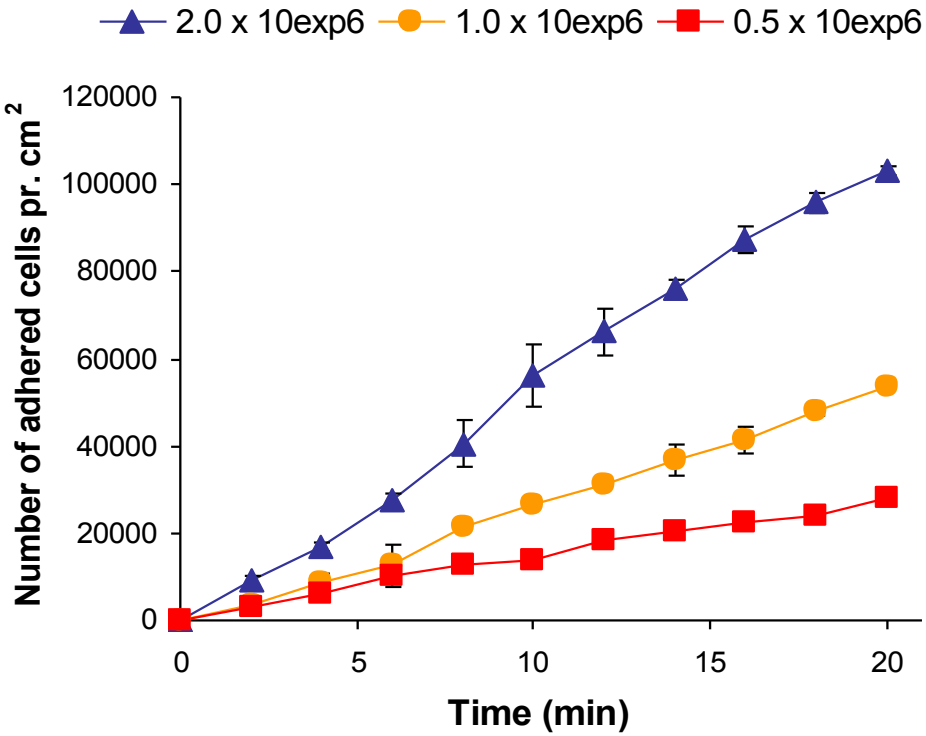
2 colour images are acquired within a few seconds

The images are converted to 8 bit grayscale images

The two 8 bit images are added to one image. A ”region of interest” is inserted and binarized with a threshold. The program is able to count the number of cells

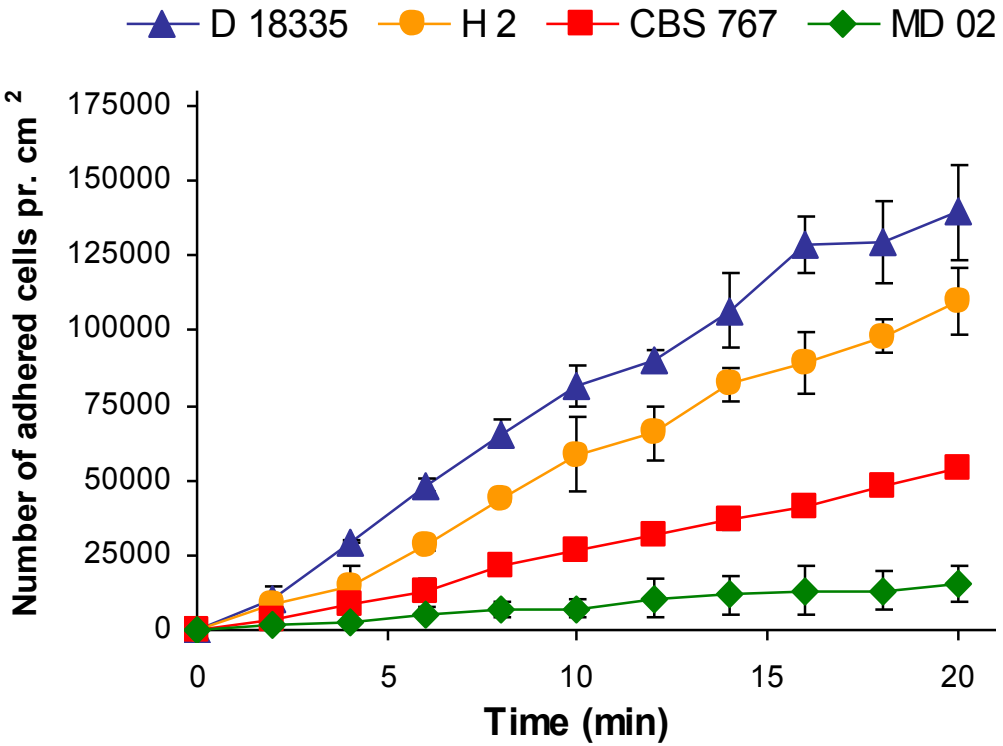


Adhesion of different cell concentrations of the *D. hansenii* type strain (CBS 767) to solid model substrate



Cell Concentration	Adhesion rate [cells·cm^{-2}·min^{-1}]
2.0 × 10^{6} cells·ml^{-1}	5445
1.0 × 10^{6} cells·ml^{-1}	**2540**
0.5 × 10^{6} cells·ml^{-1}	1355

Adhesion of different *D. hansenii* strains to solid model substrate

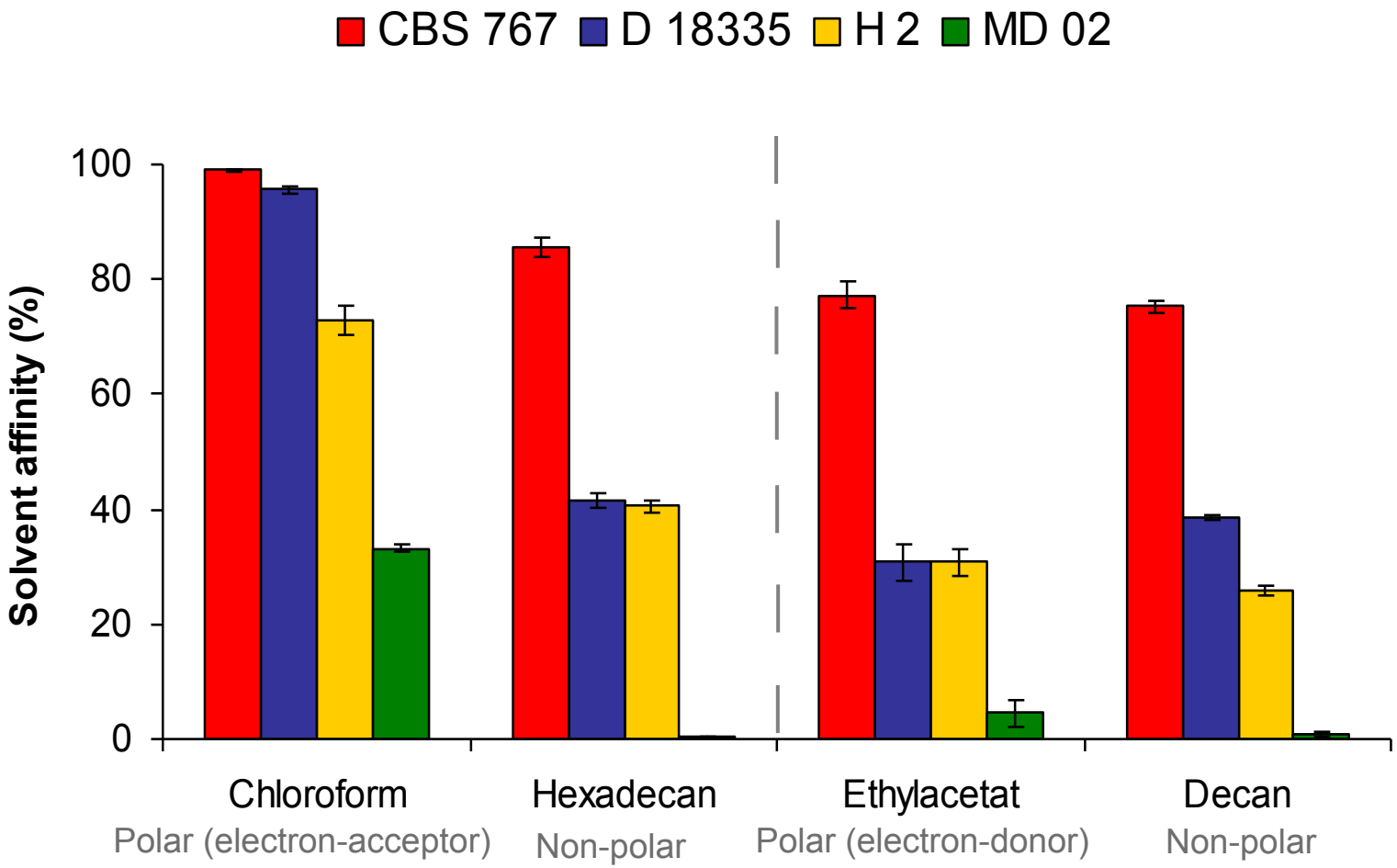


D. hansenii strains	Strain background
D 18335	Isolate from a Danbo cheese production site in Durup (K.M Petersen)
H 2	Starter culture (Danisco Cultor Innovation)
CBS 767	Type strain
MD 02	Starter culture (Arla Innovation)

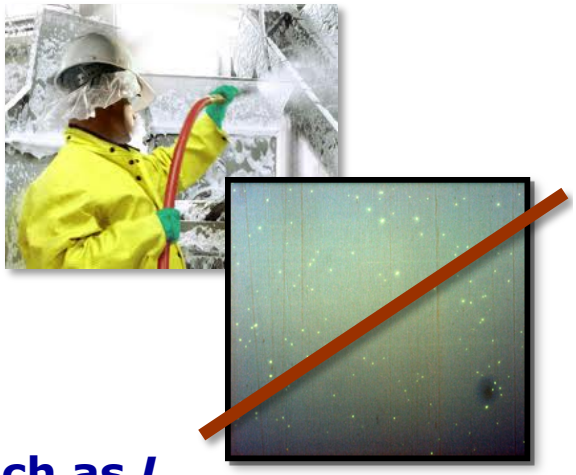
Factors of importance for cell adhesion

	Adhesion	MATS		Sedimentation	
***D. hansenii* strains**	**Adhesion rate [cells·cm^{-2}·min^{-1}]**	**Chloroform – Hexadecan [%]**	**Ethylacetate - Decan [%]**	**Avg. Cell diameter [µm]**	**Sed. rate [µm·s^{-1}]**
D 18335	**7325 (± 750)**	**53.9**	**-7.8**	**4.4 (± 0.5)**	**1.19**
H 2	**5725 (± 680)**	**32.3**	**4.9**	**3.9 (± 0.6)**	**0.91**
CBS 767	**2540 (± 360)**	**13.5**	**2.1**	**3.2 (± 0.8)**	**0.62**
MD 02	**790 (± 460)**	**33.0**	**3.7**	**3.4 (± 0.6)**	**0.70**

MATS (Microbial Adhesion To Solvents)



Why work with *L. monocytogenes* and adhesion?



- **Food safety**
- **What is of concern?**
  - **Biofilm formation - contamination of foods**
  - **Human health risk - listeriosis**
- **How to avoid or limit pathogenic bacteria such as *L. monocytogenes* in the food processing plant?**
  - **Hygienic design & good cleaning procedures**
- **Approach**
- **To limit and prevent initial adhesion and thereby biofilm formation**

Listeria monocytogenes

7 species: L. monocytogenes, L. ivanouvii, L. innocua, L. welshimeri, L. seeligeri, L. grayi and L. murrayi

Characteristics

- Rod, Gram +
- Size: *d*:0.5µm; *l*:~2µm
- Flagella (4-30°C)
- Facultative anaerobe
- Ubiquitous in nature
- Psychrophilic
  - Growth: -1.5 to 45°C
  - Optimal growth: 30-37°C
- pH: 4.0-9.6 (growth)
- Salt tolerant:10% NaCl (growth)
- A_w: > 0.95 (optimal)

RTE-foods of special concern

Listeriosis

- Fever, diarrhoea, abortion, still birth, sepsis, pneumonia, meningitis, infect nervous system
- Immunocompromised, pregnant woman, elderly
- † Mortality rate: 20-30%
- Infective dose: <1000 cells

Focus: Adhesion under dynamic flow conditions

Problematic sites contaminated with *L. monocytogenes* in the meat, poultry and seafood processing sectors (Gudbjörnsdóttir et al., 2004)

Plant	Contaminated sites	
	After cleaning	During processing
Meat sector		
I	*Trolley*	Machines, tables, scale, *trolley*, conveyer belts
IV	*Conveyer belts*	*Conveyer belts*
Poultry sector		
VII	*Cutting tables, trolleys, brining machines*	Marinade equipment, *cutting tables*, spade, *brining machine*, skinning machine, *trolley*
VIII	Crates, feather plucker, vent cutter, *tables*, trolley, *boards*	*Tables, boards*
Seafood sector		
IX	*Skinning machines*, collecting net	Slicing and *skinning machines*
X	*Conveyer belts, trays*	*Conveyer belts, trays*
XI(RTE)	Cooking equipment, flow lines	de-icing tank
XII(RTE)	*Cooking equipment*	*Cooking equipment*, forklift
XIII(RTE)	*Cooking equipment, conveyer belts, forklift*	*Cooking equipment, conveyer belts, forklift*, tubs, wood pallets

Listeria isolated from milk cheese dairies (Portugal):

- **Static**
  e.g. bench and shelves
- **Dynamic**
  e.g. milk vat and pipes

(Chamble et al. 2007; Perni et al 2007)

***L. monocytogenes* in milk processing plants in India (3 sites):**

- Raw milk collector (7/27)
- Milk silos (4/25)
- Cheese blender machine (1/27)
- Product blender (1/24)

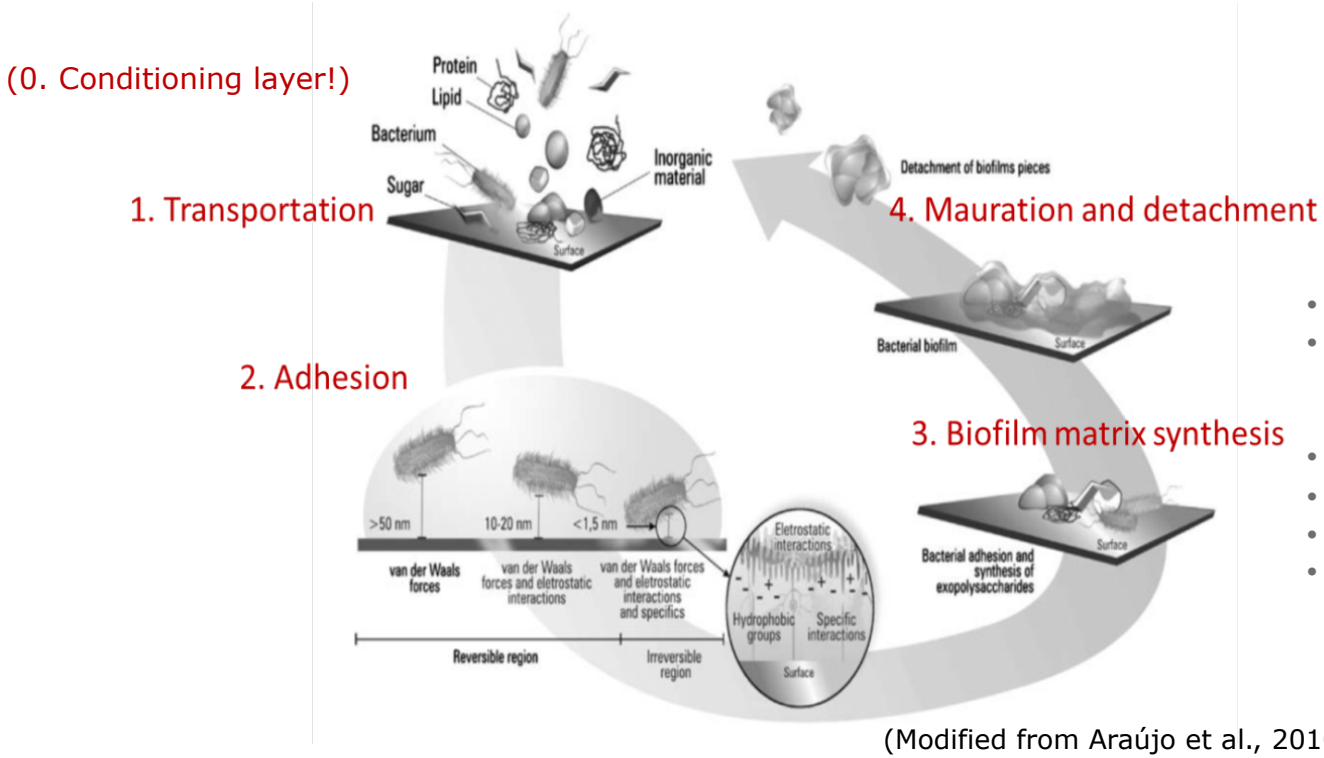
(Doijad et al., 2011)

Newly constructed commercial chicken Processing Plant:

- Raw product and drains

(Berrang et al., 2010)

Adhesion and Biofilm formation



- Water (85-90%)
- Extracellular polymer substances (EPS)
- Polysaccharides
- Protein
- Phospholipids
- Teichoic and nucleic acids ….

(Modified from Araújo et al., 2010)

Definition of Biofilm:

"a community of microbes imbedded in an organic polymer matrix, adhering to a surface"

(Carpentier & Cerf, 1993)

Definition of adhesion processes

Adhesion

”the binding together of bacteria and surface”

<u>Two distinct phases:</u>

Reversible (initial adhesion)

”The short period of time when the bacteria approaches the surface”

Irreversible (attachment)

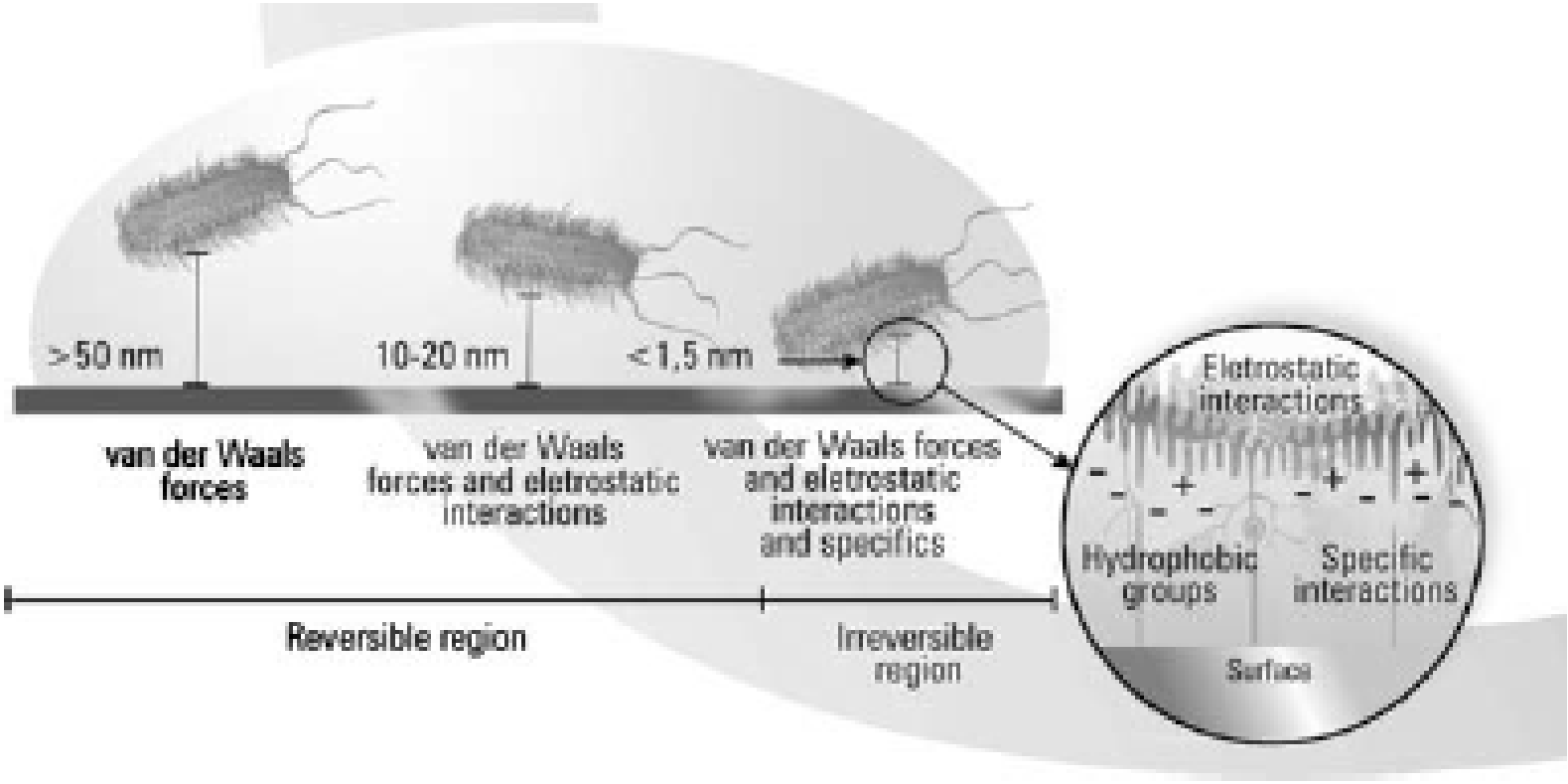
*”The bacterial cell strengthen the **attachment** to the surface”*

(Araújo et al., 2010; Deupree & Schoenfisch, 2008; Ploux et al., 2010)

Adhesion

Physicochemical interactions

(Modified from Araújo et al., 2010)

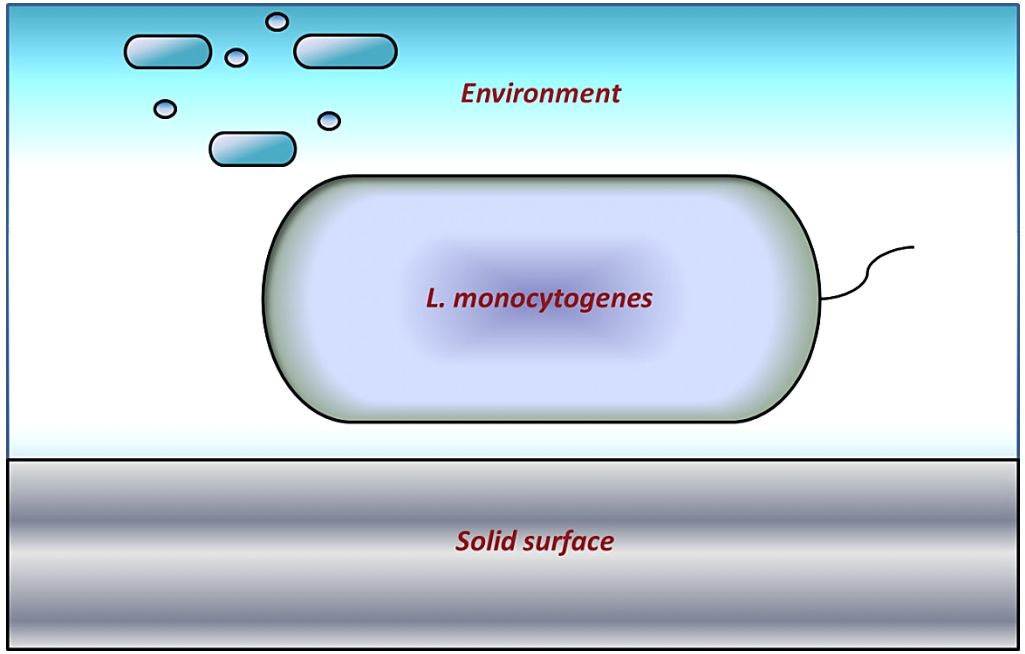


Factors involved in or influencing initial adhesion and attachment

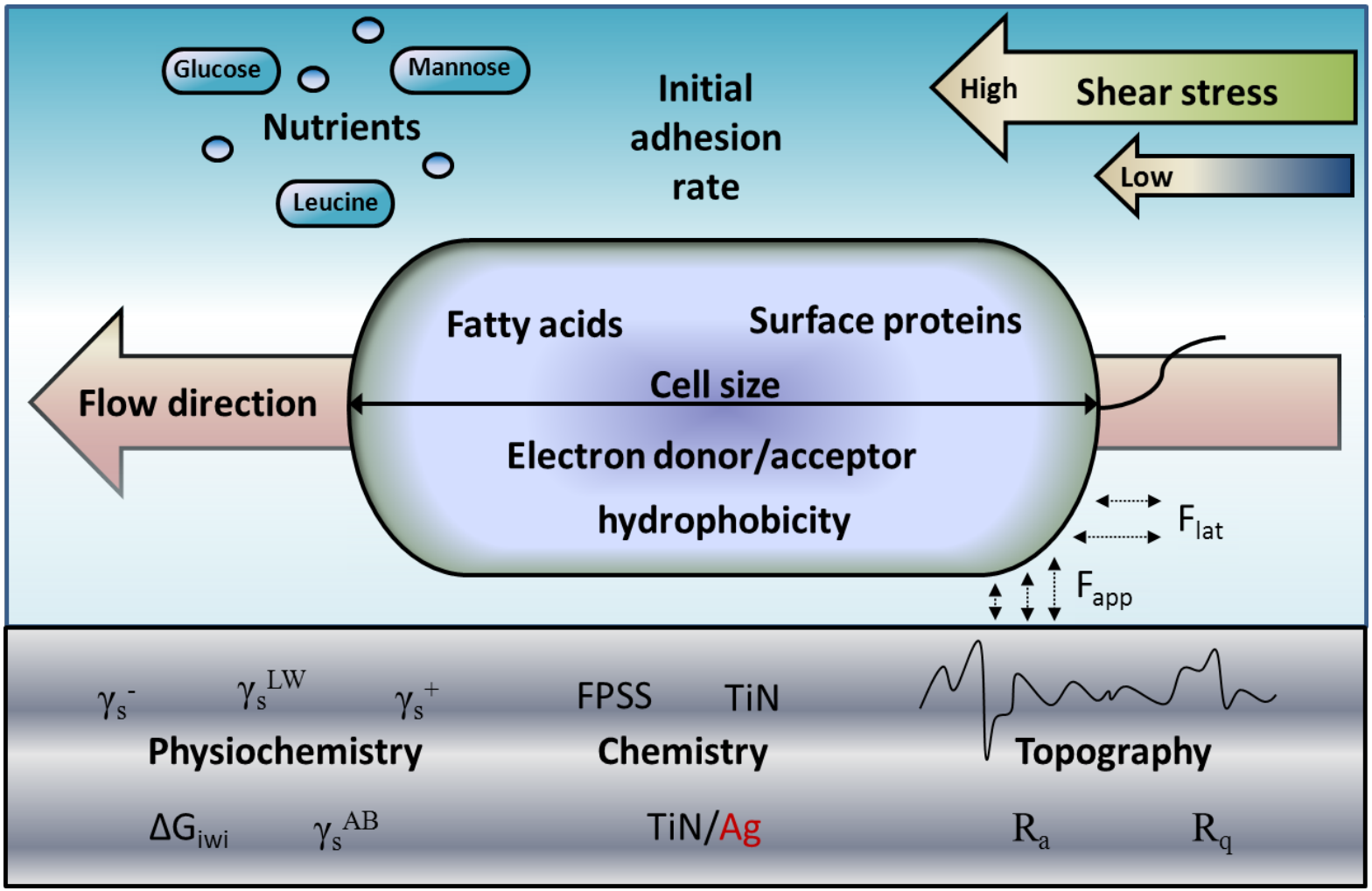
- **Bacterial surface**
- **Size**
- **Physicochemical properties**
- **Surface structures**
- **Chemistry**
- **Appendages/flagella**

- **Solid surface**
- **Topography/roughness**
- **Physicochemical properties**
- **Chemistry**

- **Environmental conditions**
- **Temperature**
- **Soil/nutrients**
- **Moisture**
- **Static/dynamic flow conditions**



Ph.D. project of Anne Skovager

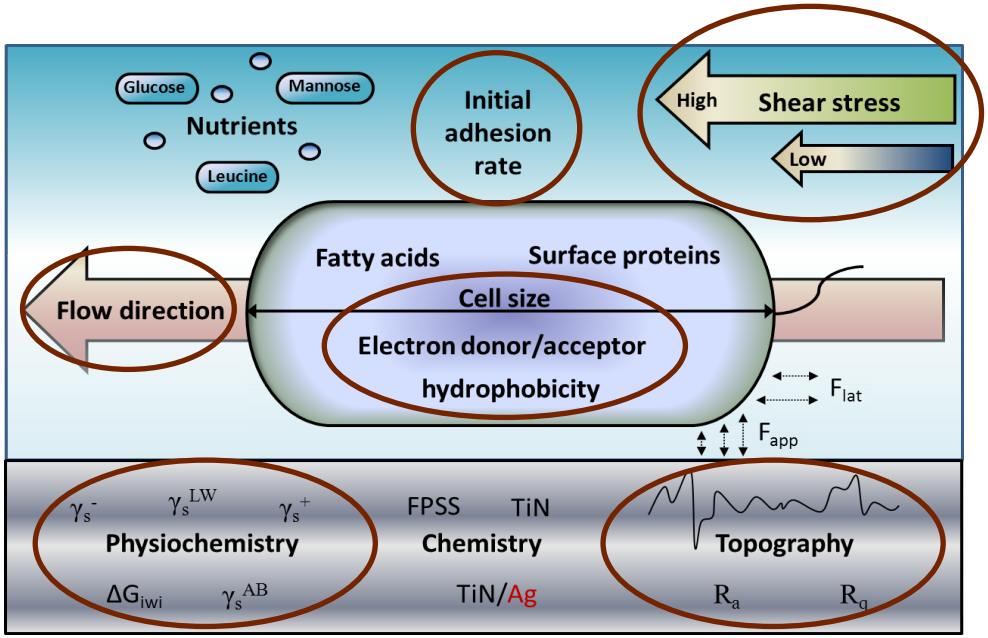


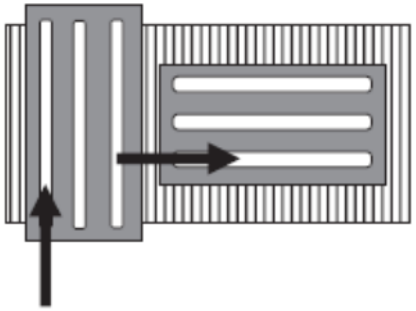
- **Aims**

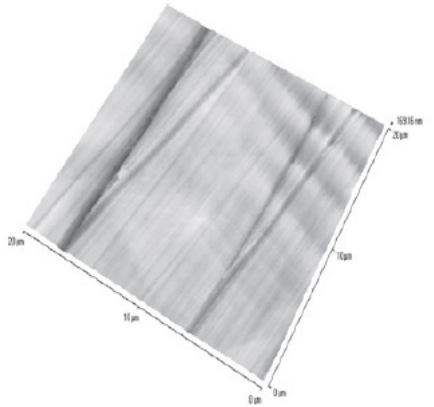
•To develop a method for detection of real time initial adhesion under flow conditions to non-transparent surfaces at single cell level.

•To examine how initial adhesion of *L. monocytogenes* to fine polished stainless steel (FPSS) is influenced by flow directions and different levels of shear stress.

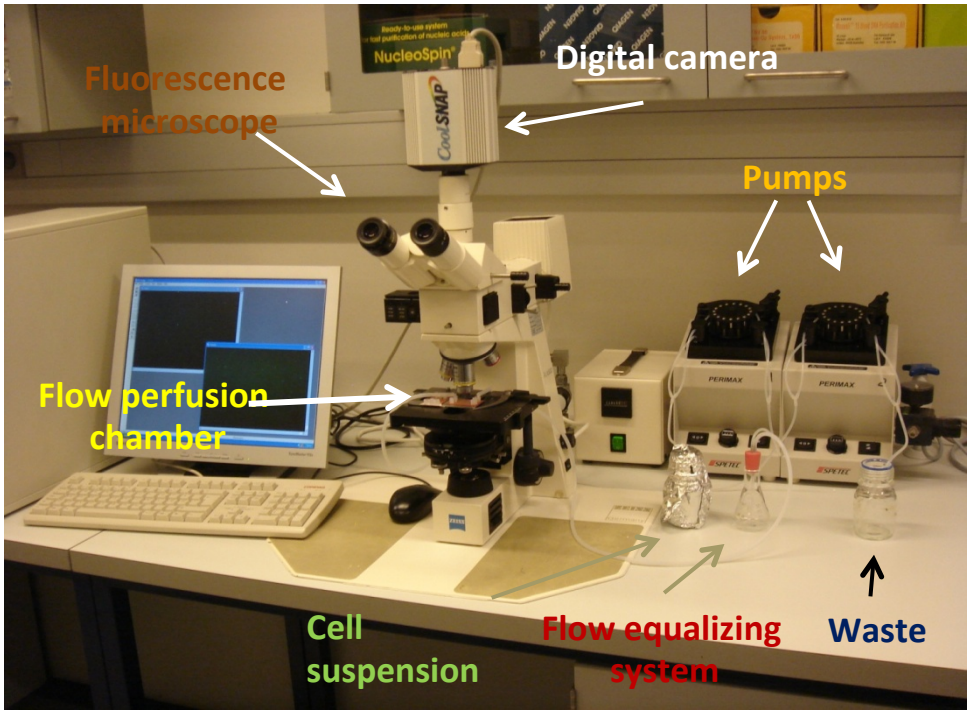
•To determine if it is possible to correlate initial adhesion rates of 7 different *L. monocytogenes strains* to cell size, and cellular physicochemical properties and surface characteristics of FPSS.

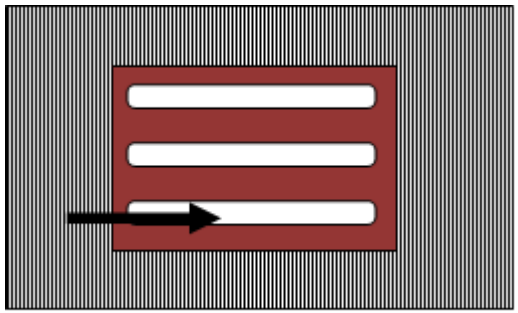






Development of method for detection of initial adhesion under flow conditions



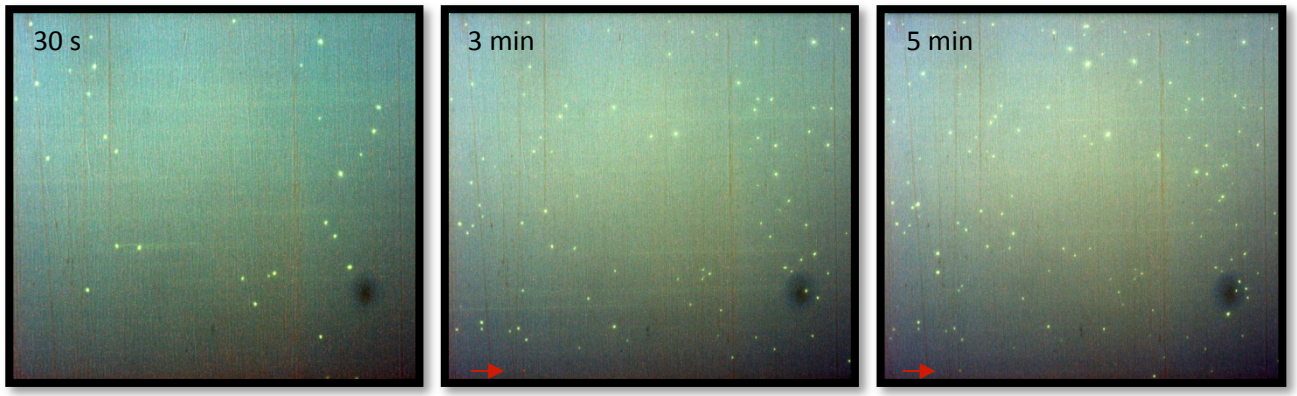


Fluorescent stain

- SYTO9 and PI (Propidium Iodine)
- GFP: Green fluorescent protein

Software for data treatment

- ImageJ



Initial adhesion rates (IAR)

cells/(min·cm^2)

Test parameters

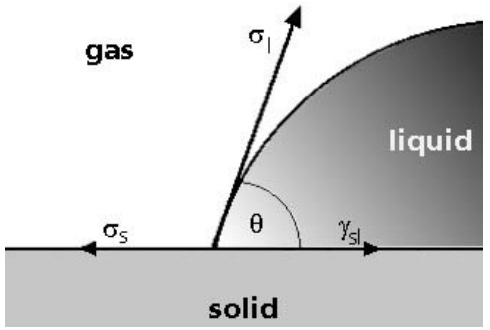
- **Flow directions: Along and across the scratches of the steel**

- **Flow rate/wall shear stress/wall shear rate:**
- **High: 8.40ml/min~1.12Pa~1400s^{-1}**
- **Low: 0.75ml/min~0.10Pa~100s^{-1}**

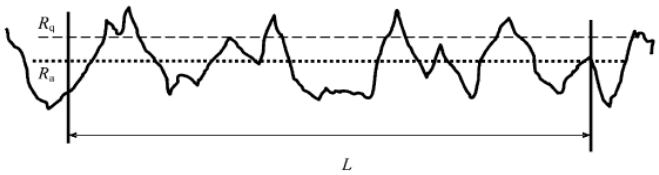
- ***L. monocytogenes* strains: EGDe (lab. strain), LO28 (Clinical), 412 (Bacon isolate), 64587/35 (splitting machine), 42222/180 (chicken chow mein), 42222/241 (curry meal), 42222/373 (chain belt line)**

- **Cellular physiochemical properties:**
- **Hydrophobicity**
- **Electron donating/accepting properties**
- **Microbial Adhesion To Solvents, MATS**

- **Cell size: Light microscopy**



- **Surface properties**
- **Physicochemical properties**
- **Contact angle measurements (CAM)**
- **Topography**
- **R_a and R_q (AFM), line-profiles (WLP)**



Initial adhesion rates and cell surface properties

Initial adhesion rates (IAR)[c] [cells/(min cm^2)]

Shear stress	0.10 Pa		1.12 Pa	
Direction[d]	Along	Across	Along	Across
EGDe	1.17 (±0.04)^{B2}	1.07 (±0.06)^{C2}	1.51 (±0.05)^{C1}	1.45 (±0.04)^{D1}
LO28	1.27 (±0.01)^{B2}	1.34 (±0.01)^{BC2}	1.72 (±0.03)^{BAC1}	1.63 (±0.04)^{DC1}
412	1.63 (±0.03)^{A21}	1.40 (±0.02)^{BA2}	1.84 (±0.08)^{BA1}	1.95 (±0.09)^{BA1}
42222/373	1.28 (±0.03)^{B2}	1.30 (±0.05)^{BC2}	1.84 (±0.05)^{BAC1}	1.83 (±0.15)^{BAC1}
42222/241	1.25 (±0.04)^{B2}	1.27 (±0.08)^{BC2}	1.79 (±0.01)^{BAC1}	1.75 (±0.05)^{BC1}
42222/180	1.18 (±0.03)^{B2}	1.13 (±0.04)^{BC2}	1.67 (±0.09)^{BC1}	1.83 (±0.06)^{BAC1}
64587/35	1.66 (±0.05)^{A2}	1.67 (±0.09)^{A2}	2.05 (±0.10)^{A1}	2.03 (±0.05)^{A1}

[a] Values with different capital letters in superscripts, within a column, were significantly different ($p<0.05$).
[b] Values with different numbers, within a row, were significantly different ($p<0.05$).
[c] The IAR values should be multiplied by 10^5. Values are means of three adhesion experiments (±standard error of the mean).
[d] Direction: flow direction across or along the scratches of the brushed stainless steel.

- **Flow direction:** No significant differences
- **Flow rate:** Significant influence on IAR
- **Strain specific:** EGDe and 64587/35
- IAR could not be correlated to cell surface characteristics

EGDe:

- Lowest IAR
- Largest cell size
- Least hydrophobic
- Most electron donating cell surface

Summary/conclusion

- **Development of new method to detection of initial adhesion under flow to non transparent surfaces at single cell level**
- **IAR was not influenced by flow direction**
- **IAR was dependent on flow rate and strain specificity**
- **EGDe showed lowest adhesion, largest cell size, least hydrophobic, most electron donating**